

Site-Directed Mutagenesis of Avibirnavirus VP4 Gene

Juan Carlos Rodríguez-Lecompte and Frederick S. B. Kibenge¹*Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island,
550 University Avenue, Charlottetown, Prince Edward Island, Canada C1A 4P3**Received July 23, 2001; returned to author for revision September 24, 2001; accepted October 9, 2001*

Virus protein VP4 of infectious bursal disease virus (IBDV) is a protease which separates VPX and VP3 from the polyprotein. We studied the importance of serine and aspartic acid on cleavage at the VPX/VP4 junction and analysed the role of the proposed H547, D590, and S653 catalytic site using five different mutations on VP4. Our results suggest that the replacement of serine by lysine in AXAAS motifs in serotype II IBDV influences polyprotein (PP) processing by VP4 and also indicate the presence of an alternative cleavage site. Mutation on D (⁵¹⁰TLAADK⁵¹⁵) prevented the cleavage at the VPX/VP4 junction, but we have found that independently of the importance of those alanines in LAA, D has an important role as part of the cleavage site. Replacement of histidine by proline H547P completely abolished PP processing. Mutation on D590 induced a partial PP processing when it was replaced by proline and the replacement of serine by proline at S653P induced a prominent change in PP processing. These results permit us to conclude that IBDV VP4 has the ability to act according to structural and topographical changes during translational and posttranslational processes and allow multiple hit sites, which serve to increase effectiveness. © 2002 Elsevier Science

INTRODUCTION

Infectious bursal disease virus (IBDV) is a member of the Birnaviridae family of the genus *Avibirnavirus* (Leong *et al.*, 2000). Viruses in this family have genomes consisting of two segments of double-stranded RNA (dsRNA), designated A and B, within a nonenveloped single-shelled icosahedral capsid. The nucleotide sequence of IBDV shows that the large segment A has a long open reading frame (ORF), and a short ORF which overlaps the 5' end of the long ORF. The long ORF encodes a polyprotein (N–VPX–VP4–VP3–C) which is processed into two structural proteins (VP2 and VP3), and the putative nonstructural (NS) viral protease VP4 (Azad *et al.*, 1985). VP4 is a proteolytic enzyme-like protein, which uses a Ser-Lys catalytic dyad to act on specific substrates and cleavage (Birghan *et al.*, 2000). It has been demonstrated that the polyprotein processing does not involve cellular proteases (Kibenge *et al.*, 1997) but involves the virus-encoded protease VP4 (Brown and Skinner, 1996), which undergoes posttranslational autocatalytic cleavage to produce VPX, VP4, and VP3 (Leong *et al.*, 2000). The exact location of the cleavage site between VPX and VP4 is not known. However, recently the presence of two alternative sites within the VPX–VP4 boundary have been suggested, which could act independently when one of them lacks functionality in *Avibirnavirus* (Sanchez and Rodriguez, 1999), as well as in

Aquabirnavirus (Petit *et al.*, 2000). In addition, it has been suggested that the MAA motifs present between VP4 and VP3 are the exact cleavage sites of this border (Sanchez and Rodriguez, 1999). Purified IBDV preparations and IBDV intracytoplasmic and intranuclear type II tubules in infected cells reacted exclusively with anti-VP4 monoclonal antibody (Granzow *et al.*, 1997). VP4 has also been shown to be a structural protein and is located at the fivefold axes of the inner layer of the IBDV capsid (Bottcher *et al.*, 1997). Mutation in VP4 could potentially affect the specificity or efficiency of cleavage of the viral polyprotein, and, by affecting polyprotein processing, such mutations might alter the virus replication rate (Brown and Skinner, 1996). Recently it has been suggested that VP4 plays a role in the activation of synthesized VP1 (Birghan *et al.*, 2000).

In the present study we induced five specific mutations on segment A cDNA of OH-IBDV and analyzed its products through a coupled *in vitro* transcription–translation reaction. The results obtained permit us to conclude that IBDV VP4 has the ability to act according to its structural and topographical changes during virus expression and also allows multiple hit sites which serves to increase its effectiveness.

RESULTS

Expression of clone pSA310

Expression of the full-length transcript of OH-IBDV segment A in clone pSA310 was confirmed by *in vitro* translation in rabbit reticulocyte lysates (Fig. 1, lane 2).

¹ To whom correspondence and reprint requests should be addressed. Fax: (902) 566-0851. E-mail: kibenge@upe.ca.

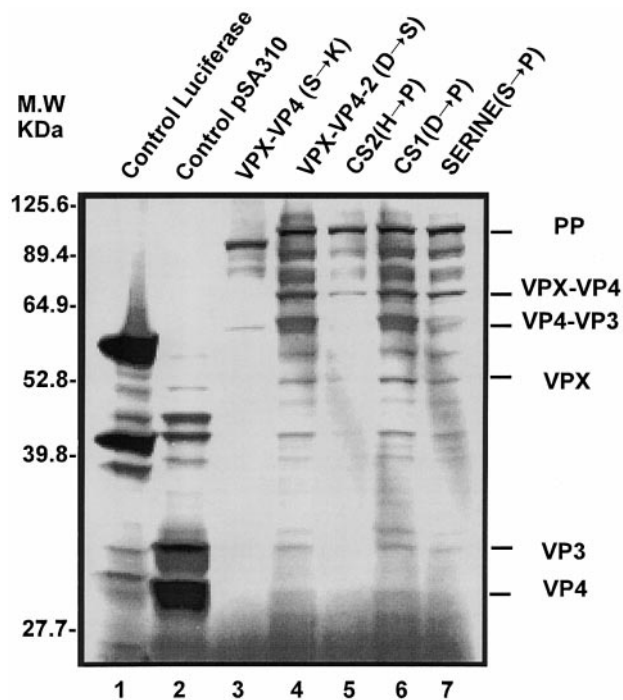


FIG. 1. Analysis of IBDV polyprotein processing products in a coupled *in vitro* transcription-translation (TNT) reaction. Lane 1 contains TNT luciferase control and lanes 2–7 contain products of pSA310, pVPX-VP4, pVPX-VP4-2, pCS2, pCS1, and pSERINE, respectively. Molecular weight marker proteins (Bio-Rad) are on the left. The identities of the viral protein products are shown on the right. In lane 5, VPX is weakly present.

This clone, which was used as the known control sample, showed the characteristic IBDV protein bands by *in vitro* translation; VPX (51 kDa), VP3 (33.5 kDa), and VP4 (29.5 kDa), which are considered to correspond to those identified in purified virions (Kibenge *et al.*, 1997). None of the five mutated plasmids showed protein bands that were similar to the wild-type control pSA310.

Cleavage site at the VPX–VP4 border of OH-IBDV by VP4

In mutated plasmid VPX–VP4, three changes were introduced at the three conserved AXAAS sequence motifs located within a 19-aa stretch at the border of VPX–VP4 ⁴⁸⁵AQAASGTARAASGKARAAS⁵⁰⁴. The serines in these motifs were mutated to lysine (K) at positions 486, 496, and 504, respectively, ⁴⁸⁵AQAASGTARAASGKARAAS⁵⁰⁴. The result shows a band corresponding to a 120 kDa polyprotein (PP) and a weak band of 81 kDa corresponding to VP4–VP3 (Fig. 1, lane 3). There were no bands such as VPX, VP3, and VP4 associated with the complete PP processing. In this case the mutations prevented full processing of PP, resulting in a partial cleavage at VPX–VP4. The other bands represent accumulation of polyproteins of aberrant molecular sizes which are commonly found in studies of PP processing in cell-free translation systems (Duncan *et al.*, 1987; Manning *et al.*, 1990; Nagy *et al.*, 1987).

In mutated plasmid VPX–VP4-2, the aspartic acid (D) in the conserved LAAD sequence motifs was replaced by serine (S) at position 514. The result showed bands analogous to PP, VPX, and VP3, but not VP4. In addition, there were three extra bands: one 81 kDa, which corresponds to VPX–VP4; one of 66 kDa, which corresponds to VP4–VP3; and one weak band corresponding to VP3 (Fig. 1, lane 4). Thus the S for D mutations produced an incomplete processing of the PP with a predominating presence of PP and VPX–VP4, more than VPX and VP4–VP3. This demonstrated that the presence of S (514) in VP4 was in some way affecting the complete processing of PP and cleavage at the VPX–VP4 border and partially the cleavage at the VP4–VP3 border.

Mutagenesis and activity of OH-IBDV protein VP4 using a catalytic triad of serine protease model

In mutated plasmid Catalytic Site 2 (CS2), histidine (H) in position 546 was replaced with proline (P) (Fig. 2). The

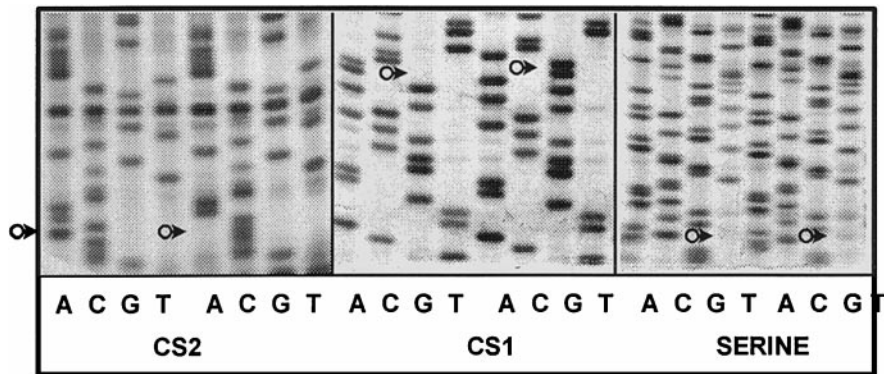


FIG. 2. Sequencing reactions to confirm site-directed mutagenesis in the VP4 catalytic site. Each panel consists of the wild-type and the mutated plasmids; mutations are identified by the respective arrows. CS2 panel has the mutated plasmid of catalytic site 2; CS1 panel has the mutated plasmid of catalytic site 1; and Serine panel has the mutated plasmid of serine as described in the text.

OH-IBDV/VP4 Wild type :

AQAASGTARGASGKARAASGRIRQLTLAADKGYEVVANMFQVQNPIVDGILASPGILRG~~GAHNL~~DCVSKEGATLFPVVITTELELTPKALNSKMFA
VIEGAREDLQPPSQRGSFIRTLSGHRVYGYAPDGVLPLETGRDYTEVPIDDVWDDSIMLSQDPIPIVGN~~SGN~~LAIAYMDVFRPKVPIHVAMTGALN
ASEIESVSFRSTKLATAHRLGMLAGPGDYDINTGPNWATFIKRFPHNPRGWDRLPYLNLPLYLPPTAGRQFHLAALAS

HHHH---H---HHHH---HHHHHHHH---HHHHH-H---E---HH---H-H---EEEE-----HHE
EHH-----EEEE---EEE-----EE-----EE---HE HHHH-----EEHHHH---
---HEEH-H-HHHHHHHHHHH---HHHH-----HHHHHHHHHH

OH-IBDV/VP4-M1/VPX-VP4

AQAASGTARGA~~K~~GKARAASGRIRQLTLAADKGYEVVANMFQVQNPIVDGILASPGILRG~~GAHNL~~DCVSKEGATLFPVVITTELELTPKALNSKMFA
VIEGAREDLQPPSQRGSFIRTLSGHRVYGYAPDGVLPLETGRDYTEVPIDDVWDDSIMLSQDPIPIVGN~~SGN~~LAIAYMDVFRPKVPIHVAMTGALN
ASEIESVSFRSTKLATAHRLGMLAGPGDYDINTGPNWATFIKRFPHNPRGWDRLPYLNLPLYLPPTAGRQFHLAALAS

HHHHHHHHHHHHHHHHHHHH---HHHHH-H---E---HH---H-H---EEEE-----HHE
EHH-----EEEE---EEE-----EE-----EE---HE HHHH-----EEHHHH---
---HEEH-H-HHHHHHHHHHH---HHHH-----HHHHHHHHHH

OH-IBDV-VP4-M2/VPX-VP4-2:

AQAASGTARGASGKARAASGRIRQLTLAADKGYEVVANMFQVQNPIVDGILASPGILRG~~GAHNL~~DCVSKEGATLFPVVITTELELTPKALNSKMFA
VIEGAREDLQPPSQRGSFIRTLSGHRVYGYAPDGVLPLETGRDYTEVPIDDVWDDSIMLSQDPIPIVGN~~SGN~~LAIAYMDVFRPKVPIHVAMTGALN
ASEIESVSFRSTKLATAHRLGMLAGPGDYDINTGPNWATFIKRFPHNPRGWDRLPYLNLPLYLPPTAGRQFHLAALAS

HHHH---H---HHHH---HHHEHHHH---HHHHH-H---E---HH---H-H---EEEE-----HHE
EHH-----EEEE---EEE-----EE-----EE---HE HHHH-----EEHHHH---
---HEEH-H-HHHHHHHHHHH---HHHH-----HHHHHHHHHH

OH-IBDV-VP4-M3/CS2:

AQAASGTARGASGKARAASGRIRQLTLAADKGYEVVANMFQVQNPIVDGILASPGILRG~~GAHNL~~DCVSKEGATLFPVVITTELELTPKALNSKMFA
VIEGAREDLQPPSQRGSFIRTLSGHRVYGYAPDGVLPLETGRDYTEVPIDDVWDDSIMLSQDPIPIVGN~~SGN~~LAIAYMDVFRPKVPIHVAMTGALN
ASEIESVSFRSTKLATAHRLGMLAGPGDYDINTGPNWATFIKRFPHNPRGWDRLPYLNLPLYLPPTAGRQFHLAALAS

HHHH---H---HHHH---HHHHHHHH---HHHHH-H---E---HH---H-H---EEEE-----HHE
EHH-----EEEE---EEE-----EE-----EE---HE HHHH-----EEHHHH---
---HEEH-H-HHHHHHHHHHH---HHHH-----HHHHHHHHHH

OH-IBDV-VP4-M4/CS1:

AQAASGTARGASGKARAASGRIRQLTLAADKGYEVVANMFQVQNPIVDGILASPGILRG~~GAHNL~~DCVSKEGATLFPVVITTELELTPKALNSKMFA
VIEGAREDLQPPSQRGSFIRTLSGHRVYGYAPDGVLPLETGRDYTEVPIDDVWDDSIMLSQDPIPIVGN~~SGN~~LAIAYMDVFRPKVPIHVAMTGALN
ASEIESVSFRSTKLATAHRLGMLAGPGDYDINTGPNWATFIKRFPHNPRGWDRLPYLNLPLYLPPTAGRQFHLAALAS

HHHH---H---HHHH---HHHHHHHH---HHHHH-H---E---HH---H-H---EEEE-----HHE
EHH-----EEEE---EEE-----EE-----EE---HE HHHH-----EEHHHH---
---HEEH-H-HHHHHHHHHHH---HHHH-----HHHHHHHHHH

OH-IBDV-VP4-M5/SERINE:

AQAASGTARGASGKARAASGRIRQLTLAADKGYEVVANMFQVQNPIVDGILASPGILRG~~GAHNL~~DCVSKEGATLFPVVITTELELTPKALNSKMFA
VIEGAREDLQPPSQRGSFIRTLSGHRVYGYAPDGVLPLETGRDYTEVPIDDVWDDSIMLSQDPIPIVGN~~SGN~~LAIAYMDVFRPKVPIHVAMTGALN
ASEIESVSFRSTKLATAHRLGMLAGPGDYDINTGPNWATFIKRFPHNPRGWDRLPYLNLPLYLPPTAGRQFHLAALAS

HHHH---H---HHHH---HHHHHHHH---HHHHH-H---E---HH---H-H---EEEE-----HHE
EHH-----EEEE---EEE-----EE-----EE---HE HHHH-----EEHHHH---
---HEEH-H-HHHHHHHHHHH---HHHH-----HHHHHHHHHH

FIG. 3. OH-IBDV VP4 amino acid sequence of the wild-type and the five different mutants in one letter code, showing the secondary structure prediction (with the program NNPREDICT). The shadowed areas refer to the five different areas of the desired mutations with the amino acid mutations identified in small letters. The secondary structure prediction H denotes α -helix and E denotes β -sheet.

results show one strong band of unprocessed PP. In addition, two weak bands corresponding to VPX-VP4 and VPX can be seen (Fig. 1, lane 5). The replacement of H by P almost completely abolished the PP processing.

In mutated plasmid Catalytic Site 1 (CS1), aspartic acid (D) in position 589 was mutated to P (Fig. 2). The result in Fig. 1, lane 6 is the same as when D was mutated to S in mutated plasmid VPX-VP4 (Fig. 1, lane 4). Once

again bands analogous to PP, VPX, and VP3, but not VP4, were observed. In addition, there were two extra bands: one of 81 kDa, which corresponds to VPX-VP4, and the other of 66 kDa, which corresponds to VP4-VP3 (Fig. 1, lane 6). Interestingly, the change on D either to S or to P affected the PP processing with the same pattern. Thus, it affected the complete processing of PP and cleavage at VPX-VP4, and partially the cleavage at VP4-VP3.

TABLE 1

Mutagenic and Selection Oligonucleotide Primers Introduced into pUC19-IBDV OH-SA and Amino Acid Substitution on VP4

Clone	Wild-type sequence	Mutated sequence	Amino acid change
M1/pVPX-VP4	5'GCACAGGCAGCCTCAGGGACGGC TCGAGGCGCGTCAGGAAAAGCCAG GGCTGCCTCAGGAAGAATAAGG3'	5'GCACAGGCAGCCAAGGGGACGG CTCGAGGCGCGAAGGGAAAAGCCAGGG CTGCCAAGGGAAGAATAAGG3'	Ser-490 → Lys Ser-497 → Lys Ser-504 → Lys
M2/pVPX-VP4-2	5'ACTCTCGCCGCTGACAAGGGGGe'	5'ACTCTCGCCGCTTCTAAGGGG3'	Asp-515 → Ser
M3/pCS2	5'GGTGCCCAACCTCGAC3'	5'GGTGCCCTAACCTCGAC3'	His-547 → Pro
M4/pCS1	5'GCACGAGAGGACCTTCAACC3'	5'GCACGAGAGCCTCTTCAACC3'	Asp-590 → Pro
M5/pSerine	5'GTGGGGAACAGCGGCAACCTG3'	5'GTGGGGAACCTGGCAACCTG3'	Ser-653 → Pro
MO/pUC19 Sspl/HpaI	5'CTTCCTTTTTCATATTATTGAAGCATTT3'	5'CTTCCTTTTTCATTAATGAAGCATTT3'	NA

In mutated plasmid Serine, S at position 652 was replaced by P (Fig. 2). The result shows bands corresponding to PP, VPX-VP4, VPX (weak), and VP3 (very weak) (Fig. 1, lane 7). In this case, the PP processing is severely affected, because it was only possible to identify clearly some intermediate products such as VPX-VP4. The weak VPX and VP3 bands suggest a fragile and incomplete processing of PP. Thus proline influences PP processing in some way, affecting the cleavage at VPX-VP4 more than at VP4-VP3.

DISCUSSION

In the present study, we analysed the protein products of OH-IBDV expressed *in vitro* during coupled transcription-translation in rabbit reticulocyte lysates after five different mutations on VP4 protease. The purpose was to evaluate the importance of serine amino acids and aspartic acid on the cleavage site at the VPX-VP4 border and also to investigate the potential role of H547, D590, and S653, a probable catalytic triad of a virus chymotrypsin-like serine protease. This is the first report of site-directed mutagenesis in IBDV.

OH-IBDV mutated plasmids VPX-VP4 and VPX-VP4-2 were created to analyse and compare the catalytic sites where VP4 protease acts in pathogenic IBDV and in IPNV and DXV (Sanchez and Rodriguez, 1999; Petit *et al.*, 2000; Birghan *et al.*, 2000; Lejal *et al.*, 2000). The VPX-VP4 clone with three mutations of serine (S) → lysine (K) yielded a 120-kDa product corresponding to the PP and 66-kDa product which matches VP4-VP3 (Fig. 1, lane 3). The VP4-VP3 weak band indicates an alternative cleavage site at the border of VPX-VP4. These results are consistent with those of Sanchez and Rodriguez, 1999, in which deletion of ⁴⁸⁵AQAASGTARAASGKARAAS⁵⁰⁴ revealed the existence of an alternative cleavage site. However, we found that the expression of VPX was totally null when we replaced all serines of the AXAAS motifs in ⁴⁸⁵AQAASGTARAASGKARAAS⁵⁰⁴, indicating the importance of the serines. On the other hand, it has been suggested that in aquabirnavirus VPX-VP4 border, cleavage by the protease occurs in Ser/Thr-X-Ala ↓ Ser/Ala-

Gly motifs, because VP4 uses the same catalytic dyad present on sequence of bacterial leader peptidases (Lon protease; Petit *et al.*, 2000). In addition, those sites are also the substrates used for herpesvirus protease (Birghan *et al.*, 2000). Hence, even though in serotype I IBDV it has been suggested that VP4 cleaves at multiple Thr/Ala-X-Ala ↓ Ala motifs (Lejal *et al.*, 2000), it is possible to infer that serotype II IBDV PP processing resembles more IPNV than serotype I IBDV.

To observe and analyse the role of aspartic acid (D) on the major substrate cleavage site (Sanchez and Rodriguez, 1999) at VPX-VP4 border, it was mutated by serine (LAAD → LAAS) in clone VPX-VP4. Three translation products were found of 105, 51, and 33.5 kDa, which corresponded to the PP, VPX, and VP3, respectively (Fig. 1, lane 4). One additional product of 81 kDa was found, which corresponded to VPX-VP4. Mutation on D affected in some way the complete PP processing influencing the cleavage of VPX-VP4. Our results are in agreement with others because the mutation in this region ⁵¹⁰TLAADK⁵¹⁵ induces incomplete processing of PP (Lejal *et al.*, 2000; Sanchez and Rodriguez, 1999), but we have found that independently of the importance of those amino acids in LAA, D has an important role, as part of the cleavage site, and surely participates actively as substrate to the correct cleavage by VP4 protease. The IPNV cleavage site between pVP2 and VP4 is situated between alanine 508 and serine 509 (Petit *et al.*, 2000), demonstrating the importance of serine, but in IBDV the composition of this part differs, by a couple of AA essential to cleavage (Lejal *et al.*, 2000). This experiment revealed that there is an alternative cleavage site in OH-IBDV at VPX-VP4, and probably it is influenced not only by ⁵¹²A ↓ A⁵¹³, but also by ⁵¹³A ↓ D⁵¹⁴ cleavage sites.

The last objective in this study was to analyse the role of H547, D590, and S653 as a probable catalytic triad of a virus chymotrypsin-like serine protease. It was decided to mutate all of them to proline to induce a hydrophobic area in this theoretical structure. In the clone CS2, PP processing was inhibited and this result is different to that reported by Petit *et al.*, 2000, who, using a wild-type

IPNV, did not observe changes in activity at the pVP2–VP4 junction, but had a strongly reduced activity at the VP4–VP3 junction when the replacement involved histidines 547 and 679 at the same time. We have observed that this mutation abolished completely the PP processing and affected directly its processing (Fig. 1, lane 5). We believe that histidine plays a very important role in the VP4 protease catalytic activity. Mutation on aspartic acid (D) in the position 589 induced a partial PP processing when it was replaced by proline (P) (Fig. 1, lane 6). Changes on D either by S or P affect the PP processing with the same results. In contrast, Petit *et al.* (2000) found that none of the mutations of conserved aspartic acid inactivated the protease. In OH-IBDV, protein VP4 was not able to act effectively like a protease. Possibly IBDV VP4 is a unique protease which has the property to act according to structural and topographical changes during translational and posttranslational processing and allows multiple hit sites to increase its effectiveness.

The mutation in this study of serine to proline at the position S652P on OH-IBDV protein VP4 induced a prominent change in PP processing (Fig. 1, lane 7). Our results are in agreement with Petit *et al.* (2000) and Lejal *et al.* (2000), who demonstrated the vital function of serine as an active residue in *Aquabirnavirus* and *Avibirnavirus* VP4 protease function. However, because we did not mutate Lys692, our results do not show the Ser-Lys catalytic dyad characteristic of Lon proteinase, but demonstrate that S652 is an essential amino acid, which if mutated, completely inactivates polyprotein processing.

MATERIALS AND METHODS

Cells and virus

The serotype 2 infectious bursal disease virus strain OH was propagated as described (Kibenge *et al.*, 1997).

RT-PCR and cDNA cloning

The methods used to obtain the complementary DNA by RT-PCR from IBDV and subsequently cloning in a pUC19 vector were used as described (Qian and Kibenge, 1994).

Site-directed mutagenesis

Sequence analysis of OH-IBDV SA using the protein secondary structure prediction program (NNPREDICT) was done to evaluate the significance of potential sites for mutation in VP4 (Fig. 3). In this study we used the Transformer Site-Directed Mutagenesis Kit (Clontech Laboratories, Inc.) with minor modifications. Briefly, the recombinant plasmid pUC-IBDV-OH was used as a template to introduce the respective mutations in the VP4 region (Table 1). One additional primer was designed to select the target plasmid DNA template; the restriction enzyme site *SspI* (position 2501) in pUC19 was mutated

with the selection oligonucleotide primer *HpaI* (Table 1). After confirmation of the presence of *HpaI* through a restriction enzyme digestion, the mutated plasmids were screened by colony blot hybridization using the oligonucleotide primers as probes labeled with [α - 32 P]dCTP. The mutations were confirmed by manual DNA sequencing using the Sanger dideoxy chain termination method (Sanger *et al.*, 1977).

Subcloning and construction of transcription vectors

Mutated plasmids were purified using a midiprep plasmid isolation method (Sambrook *et al.*, 1989). Segment A from mutated plasmids was amplified by PCR with selected primers: forward 5'TGC AAA TCA CCG CCT TGT TCC AGG3' and reverse 5'GAT CCT CAC TCC AAG TCC TCG TCA3', which included two different restriction enzyme sites *NsiI* and *BamHI*. PCR products were subcloned in a pCR 2.1 vector (Invitrogen Life Technologies). The correct orientation of the insert was evaluated by digestion with *NsiI*, which is present downstream of the T7 promoter.

Transcription/translation

We used the TNT T7 coupled Reticulocyte Lysate System (TNT-RLS, Promega) as previously described (Kibenge *et al.*, 1997). Products of these reactions were denatured and analysed by 12.5% SDS–PAGE and visualized by autoradiography at room temperature for 72 h.

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